

## Single Amino Acid Substitutions in the Hemagglutinin Can Alter the Host Range and Receptor Binding Properties of H1 Strains of Influenza A Virus

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We have previously characterized an influenza A (H1N1) virus which has host-dependent growth and receptor binding properties and have shown that a mutation which removes an oligosaccharide from the tip of the hemagglutinin (HA) by changing Asn-129 to Asp permits this virus to grow to high titer in MDBK cells. (C. M. Deom, A. J. Caton, and I. T. Schulze, *Proc. Natl. Acad. Sci. USA* 83:3771-3775, 1986). We have now isolated monoclonal antibodies specific for the mutant HA and have used escape mutants to identify alterations in HA sequence which reduce virus yields from MDBK cells without reducing those from chicken embryo fibroblasts. Two types of escape mutants which grow equally well in chicken embryo fibroblasts were obtained. Those with the parent phenotype contain Asn at residue 129 and are glycosylated at that site. Those with the mutant phenotype are unchanged at residue 129 but have a Gly to Glu substitution at residue 158, which is close to residue 129 on the HA1 subunit. Binding assays with neoglycoproteins containing *N*-acetylneuraminic acid in either  $\alpha 2,3$  or  $\alpha 2,6$  linkage to galactose showed that the MDBK-synthesized oligosaccharides at Asn-129 reduce binding to both of these receptors, leaving the HA's preference for  $\alpha 2,6$  linkages unchanged. Glu at residue 158 greatly reduces binding to both receptors without reducing virus yields from MDBK cells. We conclude that changes in the receptor binding properties of the HA can result either from direct alteration of the HA protein by host cell glycosylation or from mutations in the HA gene and that these changes generate heterogeneity that can contribute to the survival of influenza A virus populations in nature.

The influenza virus hemagglutinin (HA) is a trimeric glycoprotein which mediates both the attachment of virions to the surface of host cells and the subsequent fusion of viral and endocytotic vesicle membranes by which the transcription complex of the virus enters the cytoplasm of the host cell. It is also the virion surface protein against which neutralizing antibodies are made. The three-dimensional structure of the trimer has been determined (38), so that the regions involved in its biological functions can be identified.

A receptor-binding site, consisting of a shallow pocket into which sialic acid (SA) can be inserted, is present on the distal end of each of the three subunits which form the trimer (37, 38). The interaction of this site with SA-containing receptors on the surface of host cells is in part responsible for the host range of influenza viruses. This interaction is highly specific and can apparently be affected by the amino acid sequence in the vicinity of the receptor-binding pocket (6, 26, 34, 36), by the number and position of N-linked oligosaccharides on the tip of the HA (8, 24, 29), and by the host-determined properties of these oligosaccharides (5, 8, 9).

In an effort to expand our understanding of the role of these factors in determining the host range of the influenza A viruses, we have undertaken an investigation of mutations in the HA gene which affect receptor-binding function in a host-dependent manner. In previous work we characterized a substrain of influenza A (H1N1) virus which is restricted in its ability to grow in Madin-Darby bovine kidney (MDBK) cells although it grows well in chicken embryo fibroblasts (CEF) (5, 8). We showed that the HA1 subunit of this virus

has five glycosylation sequons, two of which are on the tip of the HA, one at amino acid 129 and a second at 184. Furthermore, mutations which remove one oligosaccharide from the tip of the HA by changing Asn-129 to Asp altered the plaque type and enhanced the ability of the mutant virus to bind to and grow in MDBK cells without changing its ability to bind to and grow in CEF. The experiments suggested that the MDBK-synthesized oligosaccharides at both positions 129 and 184 on the tip of the HA reduced the ability of the virus to bind to MDBK cells.

The experiments reported here were designed to confirm our previous findings and to determine whether the addition of a glycosylation site was required or whether other amino acid substitutions in the immediate vicinity of residue 129 would alter virus growth potential in a host-dependent manner. We postulated that monoclonal antibodies (MAbs) which neutralize the mutant but not the parent virus would select mutants with amino acid substitutions in the immediate vicinity of residue 129, independent of whether they exhibited a revertant phenotype. To this end, we have isolated MAbs which react with an epitope which includes Asp-129 of the mutant HA and have isolated spontaneous MAb-resistant mutants (MARMs) from the mutant virus population. We present here the molecular and biological properties of these MARMs, including their ability to bind to highly defined neoglycoproteins containing a single kind of SA in either  $\alpha 2,3$  or  $\alpha 2,6$  linkage to galactose.

### MATERIALS AND METHODS

Cells. MDBK cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, and

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TABLE 1. Properties of WSN HA variants<sup>a</sup>

Virus strain	Origin	Biological properties	Glycosylation sequon of HA1 at residue:		Amino acid sequence at residues 129-131
			129	184	
F <sub>0</sub>	Parent virus isolated from CEF-grown WSN stock	Fuzzy plaque type; low virus yields from MDBK cells, high from CEF; temp-dependent HA activity, low receptor-binding capacity	+	+	Asn-His-Thr
C <sub>r</sub> -1	Mutant virus selected from F <sub>0</sub> by growth in MDBK cells	Clear plaque type; high virus yields from both MDBK cells and CEF; temp-independent HA activity; high receptor-binding capacity	-	+	Asp-His-Thr
C <sub>r</sub> -10	Same as C <sub>r</sub> -1	Same as C <sub>r</sub> -1	-	+	Asn-His-Ala
C <sub>0</sub>	Isolated from the same CEF-grown WSN stock as used for F <sub>0</sub>	Same as C <sub>r</sub> -1	+	-	Asn-His-Thr

<sup>a</sup> All variants have glycosylation sequons at amino acids 20 and 21, 65, and 271 of the HA1 subunit. The sequence of the HA1 subunit of C<sub>0</sub> is identical to that of F<sub>0</sub> except that residue 146 is Ser instead of Asn and residue 184 is His instead of Asn.

primary CEF, obtained from 8- to 10-day-old embryos, were grown as described previously (5, 20).

**Preparation and assay of viruses.** The viruses used in this study are all variants of the WSN strain of influenza A (H1N1) virus which we have previously characterized (5, 8, 19, 20). Their properties are summarized in Table 1. Stocks of these viruses and of the MARMs isolated in this investigation were prepared by growing progeny from well-isolated plaques in CEF cells (5). Virus populations from MDBK or CEF cells were prepared from these stocks and purified as described by Pons and Hirst (21).

Plaque assays, hemagglutination assays, and hemagglutination inhibition (HI) tests were carried out as described previously (5, 19, 20) except that DEAE-dextran was added to the overlay medium for plaque assays at a concentration of 0.01%. Hemagglutinating units (HAU) are expressed as the reciprocal of the maximum dilution of virus causing complete agglutination of a 0.5% suspension of chicken erythrocytes. HI titers are expressed as the reciprocal of the highest dilution of antibody which inhibited 4 HAU.

**ELISA.** For enzyme-linked immunosorbent assay (ELISA), the procedure of Eisenlohr et al. (10) was used with some modification. Twelve HAU of intact virus in phosphate-buffered saline (PBS) were adsorbed to polystyrene microtiter plates by overnight incubation at 37°C. The plates were then treated for 1 h with PBS-BSA (PBS containing 1% bovine serum albumin [BSA]) at 37°C, the PBS-BSA was removed, and 50 µl of serial twofold dilutions of hybridoma fluid was added. After incubation for 2 h at 37°C, the plates were processed as previously described (10) with anti-mouse immunoglobulin G (IgG) conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.).

**Western immunoblot analysis.** Western blots were performed as described by Burnette (2). Virion proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18) and electrophoretically transferred (35) onto nitrocellulose paper with a Trans-Blot cell apparatus (Bio-Rad Laboratories, Richmond, Calif.). The paper was treated with PBS containing 1% nonfat dried milk and then with MAbs known to react with all of the variants used in this study (see Results). After incubation with anti-mouse IgG conjugated with horseradish peroxidase (Cappel Scientific, West Chester, Pa.), the HA was visualized as described previously (2).

**Production of MAbs.** Purified C<sub>r</sub>-1 virus was used follow-

ing trypsinization to remove the viral neuraminidase (30). Approximately 4,000 HAU of virus suspended in PBS was emulsified with an equal volume of Freund's complete adjuvant, and 4- to 6-week-old BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) were immunized by intraperitoneal injection. Four to six weeks later, the mice were bled and their serum antibody levels were determined by ELISA and HI. Mice with a sufficiently high antibody titer were reimmunized by intravenous injection of 500 HAU of C<sub>r</sub>-1. Three days after the second immunization, the mice were killed and their spleen cells were fused with SP2/0-AG14 myeloma cells (17) as described previously (16, 32). The hybridomas obtained from these fused cells were screened by ELISA and HI, and clones which produced HA-specific antibodies were subcloned three times to ensure stability and purity.

To obtain ascites fluid, mice were injected intraperitoneally with approximately 10<sup>7</sup> hybridoma cells 1 week after the injection of 0.5 ml of 2,6,10,14-tetramethylpentadecane (Pristane; Pfaltz and Bauer Inc., Stamford, Conn.). Ascites fluid harvested 1 week later was centrifuged at 2,000 × g for 10 min, aliquoted aseptically, and stored at -20°C.

**Binding assays.** Modified ELISAs were used to determine the binding properties of the variants. Sialyllactose (SL) covalently attached by reductive amination to human serum albumin (HSA) via an acetylphenylenediamine spacer or lactose attached by glycosidic linkage to HSA (L-HSA) via a *p*-aminophenyl spacer was used as the receptor. The SA in these neoglycoproteins (obtained from BioCarb Chemicals, Lund, Sweden) was *N*-acetylneuraminic acid (Neu5Ac). Either Neu5Acα2,3Galβ1,4Glc or Neu5Acα2,6Galβ1,4Glc was present at a concentration of 17 and 12 mol/mol of HSA, respectively. These SL-HSAs were added to polystyrene 96-well multiter plates to a concentration of 10<sup>-3</sup> nmol per well, and the plates were held at 4°C overnight. Uncoated surfaces were then treated with PBS-BSA for 30 min at 37°C, and the excess BSA was removed by extensive washing with PBS containing 0.001% sodium azide (PBS-azide). Increasing concentrations of MDBK-grown virus which had been trypsinized to remove the viral neuraminidase (30) were added to the wells in 50 µl of PBS. After incubation at 37°C for 45 min, unbound virus was removed by extensive washing with PBS-azide, and a 1:30 dilution of hybridoma fluid containing MAbs known to be equally reactive with the HA of all WSN variants was added. The plates were incubated



for 1 h at 37°C, and after thorough washing, anti-mouse IgG conjugated to alkaline phosphatase was added. The plates were again incubated for 1 h at 37°C and thoroughly washed. After addition of *p*-nitrophenyl phosphate, color production was measured at 405 nm with an ELISA reader. The amounts of anti-HA antibody and anti-mouse IgG used were those needed to ensure that the absorbance was proportional to the amount of virus bound to the neoglycoproteins. Absorbance values obtained from the binding of virus to wells containing L-HSA were subtracted from those obtained at each virus concentration.

**RNA extraction.** Approximately 100 to 200 HAU of purified virus was diluted with NTE buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA), mixed with an equal volume of proteinase K buffer (200 mM Tris-HCl [pH 7.4], 300 mM NaCl, 25 mM EDTA, 2% SDS), and incubated with 100 µg of proteinase K (Boehringer Mannheim, Indianapolis, Ind.) at 37°C for 1 h. RNA extraction and purification were carried out as described previously (23).

**cDNA synthesis.** Reverse transcription of the virion RNA was carried out as described previously (40) with a primer, designated primer I. This primer, d(AGCAAAAGCAGG), consists of a 12-nucleotide sequence which is complementary to the 3' ends of all eight segments of the influenza virus genome.

**HA gene amplification by polymerase chain reaction.** Most of the HA1 region of the HA was amplified by using Gene-Amp kits obtained from Cetus Perkin-Elmer, Inc. Reactions were carried out as described previously with primers I (see above) and IV (23). Primer IV, d(ATGTTCCTTAGTCTTGTAACCAT), is complementary to nucleotides 1045 to 1020 of the plus strand of HA DNA. Amplification was carried out for 25 cycles, each consisting of 1 min of denaturation at 94°C, 2 min at 45°C for reannealing, and 2 min at 72°C for extension. Amplified DNA was purified on 1% preparative agarose gels, electroeluted as described previously (41), and precipitated with ethanol.

**Nucleotide sequence analysis.** Double-stranded amplified DNA was denatured by treatment with 0.2 M NaOH at room temperature for 5 min, neutralized with 0.3 M ammonium acetate, and ethanol precipitated. Aliquots of this material were sequenced by the dideoxy termination method with a Sequenase kit (United States Biochemical Co., Cleveland, Ohio). DNA from the region of the HA gene which encodes amino acids 110 to 230 was sequenced directly from the amplified pool.

## RESULTS

**Isolation and characterization of MAbs directed to a defined region of the HA.** In order to isolate MAbs directed at amino acid 129 and its neighboring residues, mice were immunized with the mutant virus C<sub>r</sub>-1, which lacks an oligosaccharide at that site. The MAbs were then tested with C<sub>r</sub>-1 and the parent virus (F<sub>0</sub>) by HI and ELISA to identify those which distinguished between the mutant and the parent virus. Approximately 50 hybridoma clones produced anti-HA MAbs which recognized both viruses, whereas three produced MAbs which reacted with the mutant alone. One MAb from each group was chosen for use in the isolation and characterization of the MARMs described here.

One of the MAbs, designated 79AD2, reacted with all of the WSN variants listed in Table 1 as determined by both HI and ELISA. These MAbs recognized both native and denatured HA (Fig. 1A). They reacted with HA made in the presence of tunicamycin and with virus obtained from both

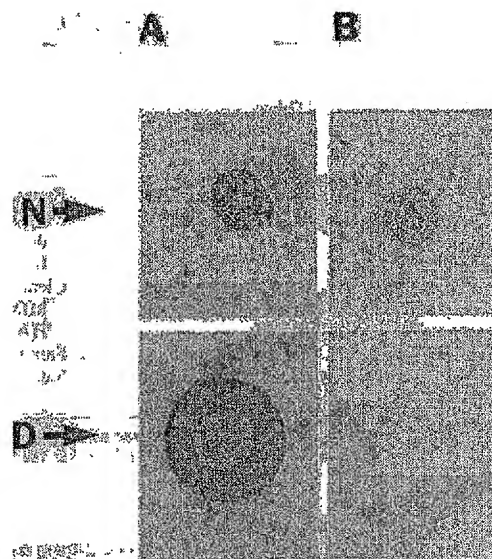


FIG. 1. Immunoblot analysis of native and denatured HA with anti-HA MAbs. Purified viral particles of the mutant C<sub>r</sub>-1 were either suspended in STE (1 M NaCl, 0.5 M Tris, 10 mM EDTA [pH 7.2]) or denatured by solubilizing in Laemmli sample buffer (18) and boiling for 5 min. Native (N) and denatured (D) viral proteins were blotted onto nitrocellulose, reacted with either MAb 79AD2 (A) or MAb 79AD8 (B), and probed with goat anti-mouse IgG antibody as described under Materials and Methods.

MDBK cells and CEF. These results suggested that the epitope recognized by these MAbs consists of amino acids that are successive in the primary structure of the HA1 and indicated that they could be used to identify the HA1 subunits from any of the WSN variants following separation of the viral proteins by SDS-PAGE.

MAb 79AD8, one of the three MAbs which reacted with the mutant but not the parent virus, reacted with the mutant HA in its native state only (Fig. 1B). Thus, the site recognized by these MAbs appeared to be a conformational epitope. This recognition site was further characterized by using the other WSN variants described in Table 1. As shown in Fig. 2, neither the parent virus (F<sub>0</sub>) nor the mutant which is glycosylated at residue 129 (C<sub>0</sub>) was recognized by the antibody. In addition to being glycosylated at residue 129, these two viruses differ from the immunizing virus by having Asn instead of Asp at position 129. C<sub>r</sub>-10, which has Asn at residue 129 but is not glycosylated because it has Ala instead of Ser at residue 131, also failed to bind the antibody. Residues 129 and 131 are in the antigenic site designated Sa (strain specific) by Caton et al. (4). Since modification of this region, either by the attachment of a carbohydrate at 129 or by amino acid substitutions at residues 129 and 131, abolished recognition of the HA, these MAbs could be used to select mutants with amino acid substitutions at or near amino acid 129 of HA1.

**Isolation of MARMs from C<sub>r</sub>-1 virus.** Virus neutralization with MAb 79AD8 was carried out by mixing approx. 10<sup>8</sup> PFU of C<sub>r</sub>-1 in 5.0 ml of isotonic saline (20) containing 0.2% BSA with an equal volume of a 1:10 dilution of ascites fluid. The mixture was incubated at room temperature for 1 h, and 1.0-ml aliquots were plated onto MDBK cell monolayers in 10-cm dishes. The infected monolayers were overlaid with a



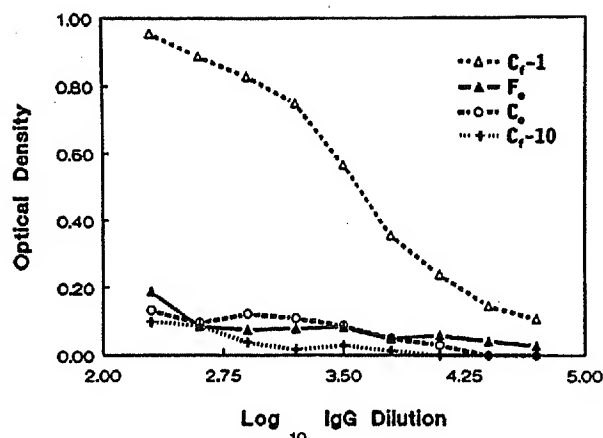


FIG. 2. Binding of MAb 79AD8 to variants of A/WSN. Assays were performed with 12 HAU of each variant and serial dilutions of ascites fluid as described under Materials and Methods.

nutrient medium containing agar and incubated for plaque development. Well-isolated plaques of both types were picked and stored at  $-70^{\circ}\text{C}$ . All plaques which were retested for sensitivity to MAb 79AD8 proved to be resistant.

Three selections were carried out, each of which was started from a separate plaque-isolated MDBK-grown preparation of  $C_I-1$ . In each experiment the two plaque types were observed at about the same frequency among the escape mutants. The frequency of mutation to antibody resistance was similar in each experiment ( $1.7 \times 10^{-6}$  to  $3.3 \times 10^{-6}$ ) and was comparable to that reported previously for the Sa antigenic site of H1 strains (11).

**Biological and structural characterization of MARMs.** Five plaques, three fuzzy and two clear, were isolated for further study. In order to avoid the characterization of sister mutants, only one plaque of each type was used from each of the three experiments. The biological properties of these isolates were compared with those of the original parent virus and of the mutant from which they were obtained. Those MARMs with fuzzy plaques ( $F_a-1$  to  $F_a-3$ ) exhibited the same restricted growth and ability to produce cytopathic effects in MDBK cells as was observed with the parent virus,  $F_0$  (Table 2). In addition, the hemagglutinating activity of the  $F_a$  variants resembled that of the parent virus in that it was lower at  $37^{\circ}\text{C}$  than at  $0^{\circ}\text{C}$ . This effect of temperature on the

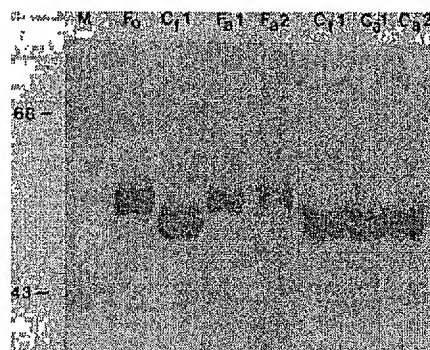


FIG. 3. Determination of the size of the HA1 subunit of MAb 79AD8-resistant mutants. Viral proteins were analyzed by SDS-PAGE, electrophoretically transferred onto nitrocellulose, and reacted with MAb 79AD2. Bound antibody was detected with goat anti-mouse IgG antibody as described under Materials and Methods. Lane M, molecular mass markers (in kilodaltons).

hemagglutinating activity of the parent virus is observed with neuraminidase-free virions and reflects the poor binding of these virions to cellular receptors (5).

The MARMs which had clear plaques ( $C_a-1$  and  $C_a-2$ ) were highly similar in growth properties and hemagglutinating activity to the mutant from which they were derived (Table 2). They are antigenic variants which have not been altered in their ability to grow in MDBK cells.

As expected from their biological properties, all three of the  $F_0$  mutants had the same amino acid substitution (Asp to Asn) at residue 129 (Table 2). This substitution restored the sequence to that of the original parent virus. The amino acid substitution responsible for the loss of recognition by MAb 79AD8 in both  $C_a$  variants is a Gly to Glu substitution at residue 158. No amino acid substitutions other than those shown in Table 2 were observed in any of the MARMs.

Western blots of the viral proteins separated by SDS-PAGE revealed that the HA1 subunits of  $F_a-1$  and  $F_a-2$  were equal in size to those of the original parent virus, indicating that the reinstated sequon at residue 129 is glycosylated (Fig. 3). As expected from their biological properties, the subunits of  $C_a-1$  and  $C_a-2$  were the same size as that of the mutant from which they were derived.

**Relative binding affinities of the MARMs for sialoglycoconjugates.** To further investigate the receptor-binding properties of the MARMs, we tested their ability to bind to

TABLE 2. Properties of MARMs

Virus strain	Plaque type <sup>a</sup>	Virus yield <sup>b</sup> (HAU/ml, $0^{\circ}\text{C}$ ) from:		Cytopathic effect in MDBK cells <sup>c</sup> (fraction of cells remaining)	HAU ratio, $0^{\circ}\text{C}/37^{\circ}\text{C}$	Amino acid at residue:	
		MDBK cells	CEF			129	158
$F_0$	F	32	512	0.55	16	Asn	Gly
$C_I-1$	C	2,048	256	0.03	<2	Asp	Gly
MARMs derived from $C_I-1$							
$F_a-1$	F	8	512	0.42	8	Asn	Gly
$F_a-2$	F	16	512	0.50	16	Asn	Gly
$F_a-3$	F	16	256	0.63	16	Asn	Gly
$C_a-1$	C	1,024	128	0.02	<2	Asp	Glu
$C_a-2$	C	2,048	256	0.05	<2	Asp	Glu

<sup>a</sup> F, fuzzy; C, clear.

<sup>b</sup> Cells were infected at a multiplicity of 0.001 PFU per cell, and maximum virus yields were determined after 36 to 48 h of incubation.

<sup>c</sup> Determined by counting trypsinized cell suspensions from monolayers at the time of infection and after 48 h at  $37^{\circ}\text{C}$ .





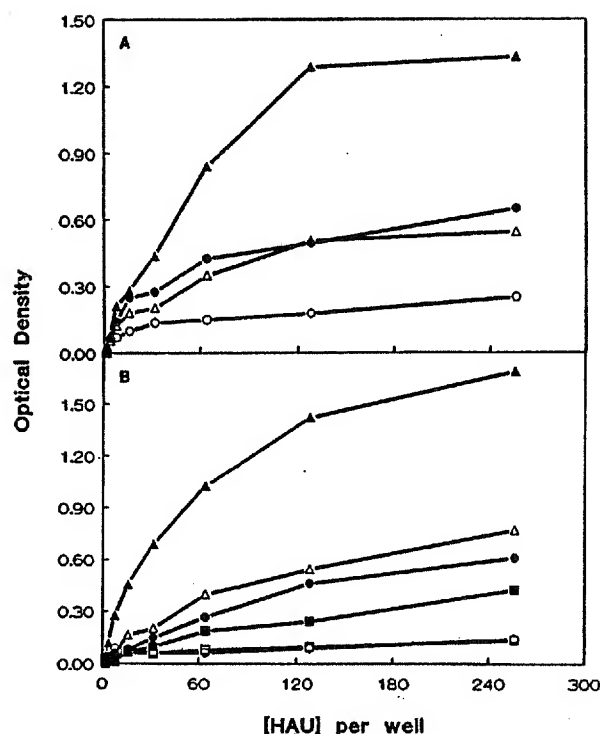


FIG. 4. Interaction of the variants with  $\alpha$ 2,3 and  $\alpha$ 2,6 SL-HSA. Virus binding was determined as described in Materials and Methods. Solid symbols,  $\alpha$ 2,6 SL-HSA; open symbols,  $\alpha$ 2,3 SL-HSA. (A) ●, ○, parent (F<sub>0</sub>); ▲, △, mutant C<sub>-1</sub>. (B) ▲, △, mutant C<sub>-1</sub>; ●, ○, MARM F<sub>-1</sub>; ■, □, MARM C<sub>-1</sub>.

neoglycoproteins containing SL covalently linked to HSA (see Materials and Methods). This assay permits measurement of binding of virus to either  $\alpha$ 2,3 or  $\alpha$ 2,6 SA-galactose sequences in the complete absence of other sialylated molecules and is sensitive enough that it can be carried out with small amounts of virus.

Figure 4A shows the interaction of the parent (F<sub>0</sub>) and the mutant (C<sub>-1</sub>) viruses with SL-HSA containing Neu5Ac $\alpha$ 2,3Gal and Neu5Ac $\alpha$ 2,6Gal. The data confirmed our previous observation that both viruses bound preferentially to Neu5Ac $\alpha$ 2,6Gal on derivatized erythrocytes (5). In addition, C<sub>-1</sub> exhibited substantially greater binding to both sialylated neoglycoproteins than F<sub>0</sub>. This was expected from our previous observation that the MDBK-grown mutant virus had a greater affinity for MDBK cell receptors than did the parent virus (5).

Figure 4B shows the binding of C<sub>-1</sub> and two of the MARMs derived from this virus to the two sialylated neoglycoproteins. MARM F<sub>-1</sub> exhibited receptor-binding properties similar to those of F<sub>0</sub>. Thus, restoration and glycosylation of the sequon at Asn 129 reduced the affinity for both sialylated neoglycoproteins to that observed with the original parent virus. In contrast, MARM C<sub>-1</sub>, which was unchanged in growth properties but has an amino acid substitution at residue 158, did not resemble the mutant virus in binding properties. Rather, it showed binding properties highly similar to those of F<sub>0</sub> (Fig. 4B). Thus, when SL containing Neu5Ac $\alpha$ 2,3Gal or Neu5Ac $\alpha$ 2,6Gal was the only sialylated molecule presented to these viruses, little binding

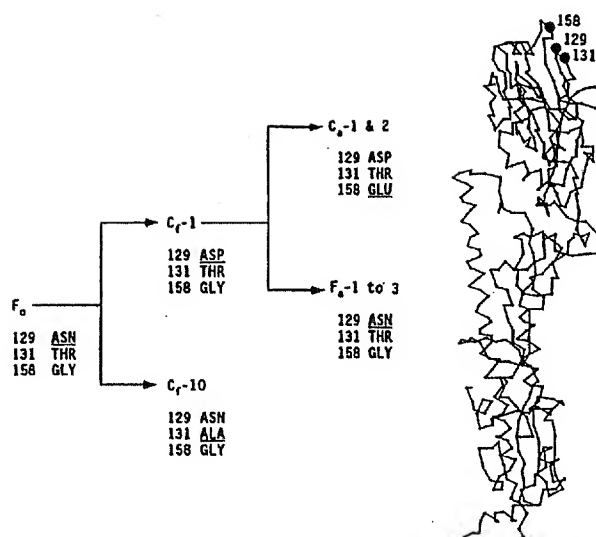


FIG. 5.  $\alpha$ -Carbon tracing of the HA monomer showing the location of the amino acid substitutions of the variants described in the text. The relationship of the variants to each other and the amino acids at residues 129, 131, and 158 in each HA are shown on the left. The underlined residues are those associated with the observed change in the biological properties of each variant. H3 numbering has been used as described previously (39).

was observed, although these viruses bound well to erythrocytes, grew to high titer in MDBK cells, and destroyed virtually all of the cells within an infected culture (Table 2). The results indicate that the Gly to Glu substitution at residue 158 causes a change in receptor affinity or specificity which can easily be detected by using highly defined binding substrates but which does not alter the ability of the virus to grow in MDBK cells.

## DISCUSSION

The MARMs which we have obtained by using an MAb directed to amino acid 129 of the HA1 subunit fall into two categories, those with a phenotype identical to that of the original parent, F<sub>0</sub>, and those which have retained the growth properties of the mutant (C<sub>0</sub>) virus from which they were derived. The MARMs from the first category are true revertants in which the glycosylation site at position 129 on the HA1 subunit has been restored. Those in the second category are not phenotypic revertants and are not glycosylated at that site. They have escaped neutralization because of a Gly to Glu substitution at residue 158.

Figure 5 presents an  $\alpha$ -carbon tracing of the HA subunit based on the three-dimensional structure of the H3 HA (38). This structure shows the proximity of residue 158 to those which constitute the glycosylation site at 129. The proximity of these residues to each other in the strains characterized here is also indicated by the fact that recognition by a single MAb is affected by amino acid substitution at either residue 129 or 158 and that denaturation of the C<sub>r</sub> HA abolishes recognition. Thus, an amino acid substitution in the immediate vicinity of residue 129 which does not reinstate the glycosylation sequon changes its antigenic properties but does not reduce the ability of the virus to grow in MDBK cells. These observations clearly implicate glycosylation at



residue 129 in restricting the growth of the  $F_0$  variant and the revertants in MDBK cells. It is clear, however, that glycosylation at residue 129 alone is not sufficient to restrict virus yields from MDBK cells, since  $C_0$  grew well in these cells (Table 1). The presence of Asn instead of His at 184 or glycosylation of that site must be involved. Site 184 is extremely close to the receptor-binding pocket.

We have previously observed that the  $C_r$ -1 mutant virus used in these studies exhibits higher affinity than the parent virus for MDBK cells as well as for erythrocytes derivatized to contain Neu5Ac linked to galactose by either  $\alpha 2,3$  or  $\alpha 3,6$  linkage (5). Since those studies also showed that the amount of viral mRNA in MDBK cells early after infection reflects the ability of the parent and mutant viruses to bind to these cells, we concluded that the increased virus yields observed with the mutant could be attributed to their increased affinity for MDBK cell receptors. The data presented here show that these differences in binding can be detected easily by using neoglycoproteins containing only Neu5Ac linked to galactose by either  $\alpha 2,3$  or  $\alpha 2,6$  linkage. Thus, with the parent and mutant viruses, both of which have Gly at residue 158, the ability to bind to Neu5Ac-containing glycoconjugates reflects the ability to bind to MDBK cells.

That is not the case with the clear-plaque-type escape mutants with a Gly to Glu substitution at residue 158. As indicated above, this amino acid substitution did not reduce the ability of the virus to grow in either MDBK cells or CEF but it did change the receptor-binding properties so that little binding was observed with either of the neoglycoproteins. One explanation for this apparent discrepancy between binding activity and ability to grow in MDBK cells is that the synthetic receptors used in the binding assays did not contain the specific SAs by which the viruses with Glu at residue 158 bind to MDBK cells. This interpretation is supported by the observation that influenza virus strains can distinguish between different sialosides and sialoglycoconjugates (12, 22, 25, 27, 33, 34) and that a number of different SAs are found on glycoproteins (28). (For example, there are five different forms of SA on bovine submaxillary protein, with Neu5Ac constituting only 37% of the total [3].) Alternatively, the amount of Neu5Ac-containing sialoglycoconjugates on the surface of MDBK cells could be sufficiently high so that HAs with low affinity for this type of SA could still bind. Such an explanation is, however, hard to reconcile with the fact that  $F_0$  exhibits low binding to MDBK cells (5) and that the  $F_0$  and  $C_a$  viruses have about the same affinity for Neu5Ac-containing glycoconjugates, as judged by their ability to bind to the neoglycoproteins used here (Fig. 4).

Gly to Glu substitutions at amino acid 158 in HA1 have been observed repeatedly during selection of MAb-resistant strains from H3 virus populations and have been isolated as natural antigenic variants of H3 HAs (6, 7, 31). This substitution is apparently important in determining whether certain H1 and H3 strains can bind to receptors on host cells. In the A/NJ/11/76 (H1N1) strain of swine virus, it is associated with a change from the L to the H phenotype, a change which is characterized by increased virus yields in chicken embryos and MDCK cells and reduced replication in the natural host (1, 13-15). An X-31 (H3N2) variant with this substitution and with a deletion of residues 224 to 230 was found to have a slight decrease in affinity for Neu5Ac $\alpha 2,6$ Gal and an increase in affinity for Neu5Ac $\alpha 2,3$ Gal when modified erythrocytes were used as receptors (7). Lastly, MAb-selected mutants with Glu at residue 158 showed lower binding to periodate-treated erythrocytes than the parent X-31 strain (36). These observations, taken together with

ours, indicate that a wide range of responses can be observed with a single amino acid substitution and stress the complexity of the receptor-HA interactions which determine host range at the cellular level.

These experiments reveal two distinct mechanisms by which the host can control influenza virus host range at the cellular level. The glycosylating enzymes of the cell can affect the structure of the HA and hence its receptor-binding properties, and the sialoglycoconjugates on the cell surface may be highly selective as receptors. The experiments also indicate that changes in the receptor-binding properties of the HA may accompany amino acid substitutions without there being detectable effects on the ability of the virus to bind to and grow in a specific host. Given the many different sialoglycoconjugates that exist in nature, heterogeneity in the HA structure generated by spontaneous mutants such as those detected here might be expected to expand the host range of the influenza virus population and foster the survival of these viruses in nature.

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